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LINOLEIC ACID EFFECTS ON EPIDERMAL DNA SYNTHESIS AND CUTANEOUS PROSTAGLANDIN LEVELS IN ESSENTIAL FATTY ACID DEFICIENCY

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An essential fatty acid (EFA) deficient state has been induced in hairless mice.

The epidermal changes included hyperkeratosis, hypergranulosis and acanthosis. Epidermal DNA synthesis was increased 3-fold compared with normal diet mice. Prostaglandin E (PGE) and prostaglandin F (PGF) levels, measured by radioimmunoassay, were much reduced in the EFA deficient mice skin.

10% Linoleic acid applied topically for 2 weeks corrected the gross and histological skin abnormalities and reduced epidermal DNA synthesis to normal values. The levels of PGE and PGF were only partially corrected. Linoleic acid applied to normal diet mice increased skin levels of PGE and PGF compared with the control vehicle treated normal diet mice.

These results provide further evidence for the importance of essential fatty acids in the control of epidermal proliferation and differentiation. The importance of PGE and PGF in controlling epidermal DNA synthesis in EFA deficiency is less clear.

The cutaneous features of animals fed an essential fatty acid deficient diet have been known for some time [1]. In addition to scaling and thickening of the skin in the nonhairy areas, hair loss has been noted after a variable time on the diets. It has been previously shown that EFA deficient hairless mice have an increased epidermal DNA synthesis and that it is possible to correct this abnormality by the application of linoleic acid [2].

Previous studies of the epidermal mitotic counts resulted in confusing data, some workers finding normal mitotic count [3], but Menton found a high mitotic index in haired mice on an EFA deficient diet [4]. Kingery and Kellum [5] noted an increased mitotic activity in EFA deficient rats but did not make actual mitotic counts. Nasr and Shostak [6] showed further conflicting results on mitotic activity in the skin of EFA deficient haired mice. Their female animals showed a raised mitotic index but their male animals did not.

Adult patients in a state of EFA deficiency developed a chronic scaling dermatosis and it has been noted that the application of linoleic acid lessens the degree of this scaling [7]. It has also been shown that topical prostaglandin E_2 (PGE_2) lessens the scaling of EFA deficient rats and that the skin of these rats showed impaired synthesis of PGE_2 [8].

Disturbed epidermal sterol metabolism [9] and prostaglandin levels [10,11] have recently been found in patients with psoriasis. Though these disturbances are different from that found in EFA deficient skin [7], it is possible that disturbed cutaneous lipids are associated with altered epidermal cell production and differentiation.

It was therefore felt important to investigate any correlation between epidermal DNA synthesis and prostaglandin levels in the experimental epidermal proliferation of EFA deficiency and to see if linoleic acid, an EFA precursor of prostaglandins, would correct any disturbances of prostaglandin levels in addition to correcting the cutaneous abnormalities.

MATERIALS AND METHODS

All reagents were of analytical grade. Tritiated PGE and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) assay kits were used for the measurement of PGE and PGF in skin extracts (Clinical Assays, Inc., Cambridge, Massachusetts). Tritiated PGE_2 (specific activity 117 Ci/mMol) was purchased from New England Nuclear, Boston, Massachusetts. Silicic acid (60–200 mesh) and linoleic acid were purchased from Sigma Chemical Company, St. Louis, Missouri. Linoleic acid was stored at 0°C and made into a 10% solution in a vehicle of propylene glycol:ethanol (3:7) immediately before use.

The EFA deficient diet was obtained from Sprague-Dawley diet division, Madison, Wisconsin [2]. The standard diet, Wayne Lab Box, was obtained from Allied Mills, Inc., Chicago, Illinois.

Animals

Female hairless mice (HRS/J strain) were obtained at 3 weeks of age. The mice were randomly separated into 2 groups. One group was fed the EFA deficient diet, the other group was fed the normal diet.

At 50 diet days the EFA deficient mice and the normal diet mice were randomly separated into groups. One group of EFA deficient and 1 group of normal diet mice were treated daily with 20 μl 10% linoleic acid to the back skin for 14 days. The other mice were treated with 20 μl of the vehicle. All mice were immobilized for 30 mins to minimize the removal of the topical solution, each mouse being caged separately to reduce ingestion of the solutions. Female mice were used since they did not fight and thus their skin was undamaged at the start of the experiments.

Skin for Prostaglandin Assays

It was decided to measure PGE and PGF levels in untreated skin because it had been noted previously that epidermal DNA synthesis in the distant skin of EFA deficient mice treated with topical 10% linoleic acid returned to normal diet values, whereas locally treated areas remained slightly raised [12]. This may have been because of primary irritation or frictional stimulus from the linoleic acid application, and the irritation or friction may have produced a local disturbance of prostaglandin formation.

At 65 diet days skin samples were taken from untreated neck areas of the mice. They were snap frozen in liquid nitrogen and stored at −20°C for approximately 3 weeks. Immediately before homogenization these skin samples were placed on blocks of carbon dioxide ice. Samples were cut with a 10-mm punch and were weighed while still frozen.

These skin were homogenized for 30 sec in an ice-cold solution of 1 ml phosphate buffered saline and 3 ml ethyl acetate:isopropanol: 0.1 M HCl (3:3:1 v/v/v) using a Brinkman PT 10 ST homogenizer. Samples were kept in an ice bath throughout homogenization.

The sample tubes were set at room temperature and mixed every 2 min for 10 sec over a 10-min time period. Two ml ethyl acetate and 3 ml H_2O were then added, followed by final mixing, and the mixture was centrifuged for 5 min at 1600 ×g. The organic phase was removed and dried at 55°C in an airstream. Samples were stored at −20°C until purification.

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Abbreviations:
EFA: essential fatty acid
PGE: prostaglandin E
PGF: prostaglandin F

Purification and separation of the PG fractions was performed using silicic acid column chromatography [12].

The recovery of PGE from the columns was in the S4 solvent (benzene:ethyl acetate:methanol, 60:40:2) and was estimated by running three additional columns which were run with known amounts of $^3HPGE_2$.

The recovery of PGF was in the S2 solvent (Benzene:ethyl alcohol:methanol, 60:40:20) and the recovery efficiency was estimated in the same way as for PGE described above. Blank columns (1 for PGE and 1 for PGF) were also run and the fractions collected in the S4 and S2 solvents were later included in the PG assays.

Radioimmunoassays

Determination of PGE and PGF content was measured by commercially available kits based on modifications of the methods of Jaffee and Behrman [13], Levine, Gutierrez-Cernosek, and Van Vunakas [14], Gutierrez-Cernosek, Morril, and Levine [15] and Caldwell, Burnstein, and Brock [16] and followed instructions accompanying the radioimmunoassay kits.

Purified PGE was resolubilized in 1 ml Tris HCl NaCl-gelatin (Isogel) buffer and converted respectively to total PGB (i.e., $PGB_1 + PGB_2$) by alkaline treatment at 100°C as instructed. The PGB standards and unknown biological samples were incubated with rabbit anti-PGB_1 serum (sensitive to both PGB_1 and PGB_2) for 1 hr at 37°C. The radioimmunoassays were carried out in duplicate. Separation of the bound antigen from the free prostaglandin was achieved by precipitation with goat antirabbit serum for 18 hr at 4°C. After centrifugation at 1600 × g for 30 mins the supernatant was poured off and the bound material resolubilized with 0.1 N NaOH and radioactivity measured in a Beckman LS-150 liquid scintillation counter.

Known quantities of tritiated PGE_2 (5000 cpm) had been added to each tissue sample, and PGE levels were determined from the standard curve (range of 8 pg to 2 ng) and then corrected to the original volume and content as a function of both the percent recovery (60.1 ± SEM 3.7%, n = 3) of tritiated PGE_2 added to representative silicic acid columns and of the percent conversion of purified PGE to PGB by alkaline treatment (74.6 ± SEM 5.2%, n = 3). The latter fraction was determined by reextraction and purification of known quantities of tritiated PGE that were treated by alkalinization at the same time as the tissue samples. The unknown samples of tissue homogenate yielded a response curve parallel to the curves.

The PGF fraction of the silicic acid column was resolubilized in the Isogel buffer. Known quantities of tritiated $PGF_{2\alpha}$ (5000 cpm) had been added to each tissue sample, and their recovery calculated from the standard curve.

A standard curve was established using known amounts of $PGF_{2\alpha}$ over a range of 9.4 pg to 2.4 ng

PGF levels were determined from this standard curve and then corrected to the original volume and content as a function of the percentage recovery from the silicic acid column (61.6 ± SEM 4.9% n = 3, samples).

It is likely with these radioimmunoassay kits that values of PGE and PGF may be partially inaccurate. Care was taken to separate PGE from PGF using the silicic acid column but it should be stressed that the radioimmunoassays do not give specific separation for PGE_1 versus PGE_2, $PGF_{1\alpha}$ versus $PGF_{2\alpha}$ or PGA versus PGB, there being significant antisera crossreactivity for the above groups of PG's.

In Vivo Epidermal DNA Synthesis

The mice were injected intramuscularly with 25 μCi of tritiated thymidine immediately after the biopsies for PG measurement were obtained.

One hour later the animals were sacrificed by ether inhalation, skin samples from treated (back) and distant (abdomen) sites were taken. Punch biopsies for autoradiography were removed and the remainder of the skin heated at 55°C for 2 min to facilitate removal of the epidermis by scraping. Epidermal samples were stored at −20°C until hydroxylapatite column DNA extraction.

Measurement of DNA Synthesis

The hydroxylapatite column extraction of epidermal DNA followed the procedure described by du Vivier et al [17]. Epidermal samples were homogenized with a Brinkman PT10ST, extracted, and measured by spectrophotometer and liquid scintillation counter. The results were expressed as counts per minute per 1 μg DNA (cpm/μg).

Autoradiography

Biopsies were taken from the animals at the same time as epidermal samples were obtained for hydroxylapatite column extraction of DNA. The biopsies were fixed in neutral formal solution, sections were cut at 4 μ, deparaffinized and dipped in Kodak nuclear track emulsion NTB-2. The dipped slides were then developed after 3 weeks exposure in light tight boxes at 4°C using Kodak D19 developer, followed by Edwal Quick Fixer. The slides were then stained with hematoxylin and eosin and counted without knowledge of the status of the biopsy specimen.

Epidermal cells containing more than 5 grains were counted in the interfollicular epidermis and the labeling index expressed as the number of labeled basal cells per 1000 epidermal basal cells.

RESULTS

Gross Cutaneous Observations

The skin gradually showed a change in the EFA deficient animals that began with a loss of normal skin elasticity. After 40–50 days on the diet there was an increase in the skin creases and a diffuse thickening of the skin with fine scaling and increased shininess.

In the EFA deficient mice treated with linoleic acid there was an initial increased skin scaling followed after about 10 days by a return of the skin appearance to those of normal diet mice.

There were no gross changes seen in normal diet mice treated with linoleic acid.

Prostaglandin Skin Levels

EFA deficient mice treated with the control solution had a marked reduction of PGE and PGF. These are summarized in Table I.

EFA deficient mice treated with linoleic acid showed a significantly increased concentration of PGE and PGF over the control group. The levels were significantly reduced when compared to normal diet mice.

Normal diet mice treated with linoleic acid had significantly higher levels of both PGE and PGF when compared to the normal diet control vehicle mice.

Epidermal DNA Synthesis

The 65 diet day EFA deficient mice treated with control solution showed a significantly increased epidermal DNA synthesis compared to the normal diet, control vehicle treated mice. Autoradiographic labeling indices showed a high labeling index with labeled cells confined to the lower 2 epidermal cell layers (Figure 1*a*).

Linoleic acid treated EFA deficient mice showed a complete return to normal DNA synthesis in distant skin but locally treated skin remained slightly higher than normal. This may signify some primary irritant action by the linoleic acid (see also the linoleic acid treated normal diet mice skin). These results are summarized in Table II.

TABLE I. *Cutaneous PGE and PGF values in EFA deficient or normal mice treated with linoleic acid*

	PGE *ng/gm skin ± SEM*	PGF *ng/gm skin ± SEM*
1. EFA deficient	17.8 ± 2.6	2.7 ± 0.3
2. EFA deficient + linoleic acid	33.1 ± 4.7	6.6 ± 1.6
3. Normal	108.1 ± 19.2	93.6 ± 23.0
4. Normal + linoleic acid	272.2 ± 23.8	191.3 ± 25.4

n = 6 mice in each experimental group.
p values: 1. PGE v's 2. PGE $p < 0.02$
1. PGF v's 2. PGF $p < 0.02$
1. PGE v's 3. PGE $p < 0.01$
1. PGF v's 3. PGF $p < 0.01$
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3. PGE v's 4. PGE $p < 0.01$
3. PGF v's 4. PGF $p < 0.01$

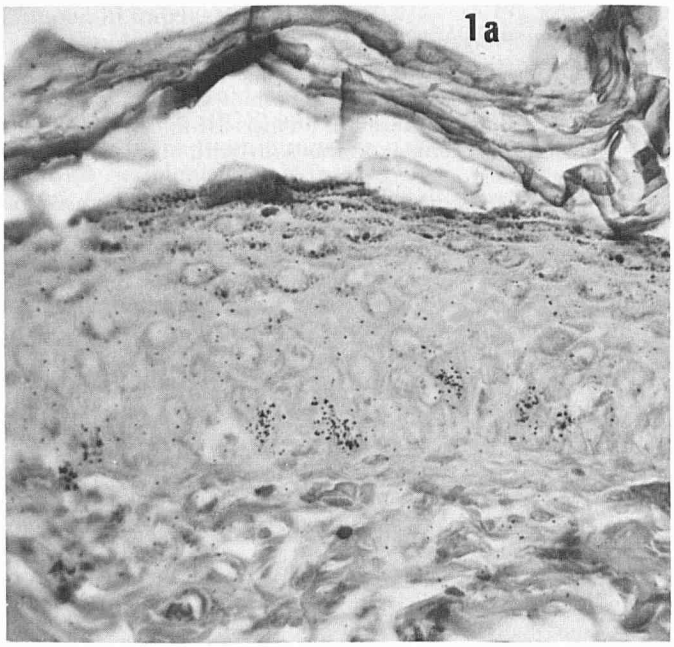
Normal diet mice treated with linoleic acid also showed a slight increase in DNA synthesis in treated sites (probably primary irritation) compared with control vehicle treated sites. Distant sites were not significantly different from controls.

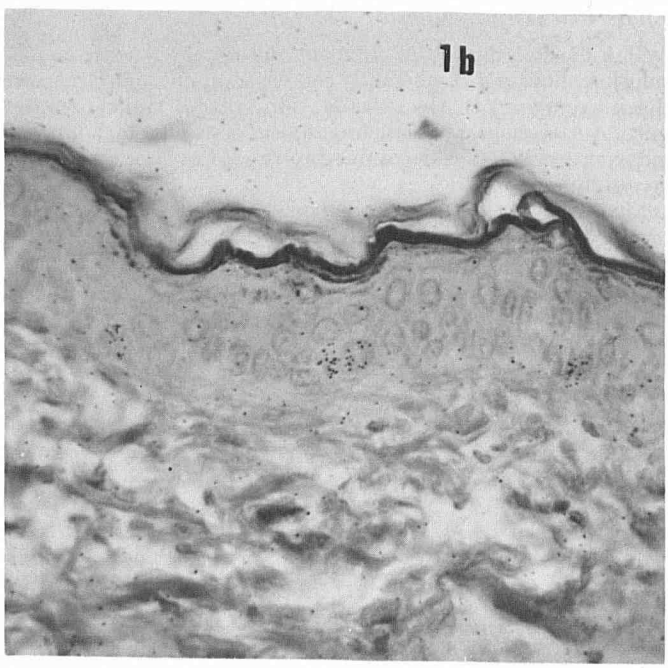
Skin Histology

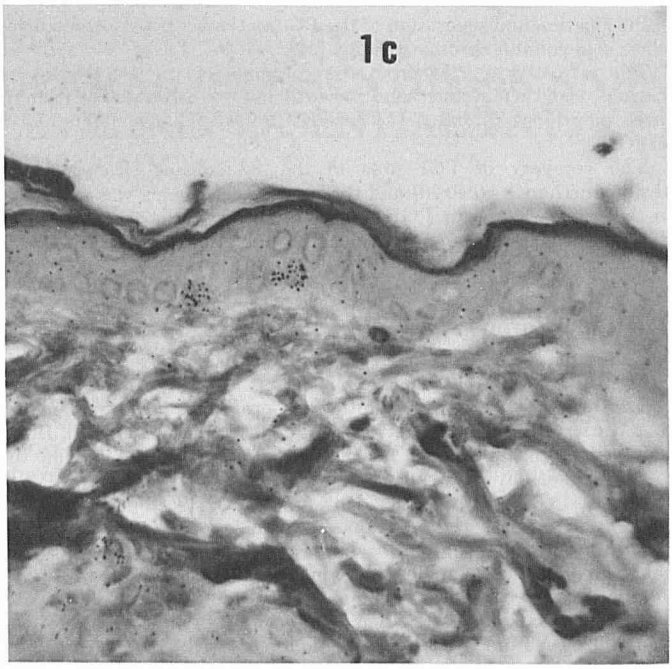
The EFA deficient epidermis was characterized by acanthosis, hyperkeratosis and hypergranulosis. There were increased intraepidermal cell spaces and vacuolated epidermal cells were seen (Figure 1*a*).

The linoleic acid treated EFA deficient mice epidermis showed a return to nearly normal epidermal thickness. There was a reduction of hyperkeratosis, hypergranulosis and labeling indices (Figure 1*b*).

Normal diet mice skin is shown (Figure 1*c*) and there were no significant changes in linoleic acid treated normal diet mice skin histologically.







Autoradiographs × 450 stained with H&E. All biopsied 1 hr after tritiated thymidine. 1*a*, EFA deficient mouse skin treated with control vehicle. Note acanthosis, hypergranulosis, hyperkeratosis and heavy labeling of lower 2 epidermal layers. 1*b*, EFA deficient mice skin treated with topical 10% linoleic acid daily for 14 days. The epidermis has almost returned to the thickness of normal diet mice and there are fewer labeled basal cells. 1*C*, normal diet mice skin.

DISCUSSION

The importance of a normal lipid profile to ensure normal keratinization may have been concluded from the descriptions of fatty acid deficient animals by Burr and Burr [1].

The cutaneous abnormalities in EFA deficiency probably involve several mechanisms. Essential fatty acids may be directly or indirectly required for normal keratinization and control of epidermopoiesis.

Adult patients in a state of EFA deficiency develop a chronic scaling dermatosis and it has been noted that the application of linoleic acid in sunflower seed oil lessens the degree of scaling of these EFA deficient humans [7].

Ziboh and Hsia [8] showed the epidermis of EFA deficient rats to have increased monoenoic and eicosatetraenoic acids and decreased dienoic and tetraenoic acid. They showed clearing of the skin scaling in the EFA deficient rats when the animals were treated with topical PGE_2. They also found that in EFA deficiency there was elevation of an abnormal fatty acid w9, 5:8:11 eicosatrienoic acid. They considered it likely that this w9 acid inhibits cyclooxygenase activity which would inhibit the conversion of arachidonic acid to PG precursors. The w9 eicosatrienoic acid is only present in minute amounts in normal skin [18], but in EFA deficiency the enzymes that would normally convert linoleic acid to arachidonic acid instead convert the oleic acid to the w9 eicosatrienoic acid [19]. This w9 acid is not converted to prostaglandins. Part of the reason for lowered PG's is likely to be reduced amounts of precursor arachidonic acid in EFA deficiency [20]. De Thomas, Brenner, and Peluffo [21] have shown that the w9, 5:8:11 eicosatrienoic acid substitutes for arachidonic acid in phospholipids. It is possible that the accumulation of the abnormal w9 acid leads to the defective skin barrier function seen in EFA deficiency [7]. Increased skin PG levels were found in linoleic acid treated normal diet mice and may have been the results of increased amounts of precursor linoleic acid plus normal enzyme conversion to PG's.

PGE compounds have however also been shown to increase epidermal DNA synthesis when provided exogenously. Bem and Greaves [22] showed that PGE_1 in concentrations below 20 μg/ml increased epidermal DNA synthesis and skin thickness

TABLE II. *Epidermal DNA synthesis (SA) as cpm/μg DNA and labeling indices (LI) per 1000 epidermal basal cells in hairless mice treated with 10% linoleic acid*

Agent	Treated Skin		Distant Skin	
	Mean SA ± SEM	*Mean LI ± SEM*	*Mean SA ± SEM*	*Mean LI ± SEM*
EFA deficient + 10% linoleic acid daily for 14 days n = 8	53.0 ± 2.7	68.0 ± 18.3	35 ± 3.6	61 ± 16.1
EFA deficient + control vehicle daily for 14 days n = 6	98 ± 7.5 $p < 0.01$	253 ± 12.5 $p < 0.01$	95 ± 6.5 $p < 0.01$	234 ± 13.2 $p < 0.01$
Normal + 10% linoleic acid daily for 14 days n = 6	49.6 ± 6.0	—	43.2 ± 5.5	—
Normal + control vehicle daily for 14 days n = 6	38.9 ± 6.5 $p > 0.05$	—	37.4 ± 3.7 $p > 0.05$	—

with mouse skin in vitro. Eaglestein and Weinstein [23] showed that intradermal injections of 1 μg of PGE_2 in humans produced increased epidermal DNA synthesis after 24–48 hours. Lupulescu [24] showed that intramuscular injections of PGE_1 and PGE_2 increased incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine in rat skin. $PGF_{2\alpha}$ decreased incorporation of these tritiated precursors [24]. A synthetic PGE_2 methyl ester has also been shown to increase epidermal DNA synthesis in normal mouse skin [25]. It may be that a correct balance of epidermal prostaglandins is more important than absolute concentrations in the normal control of epidermal proliferation and differentiation.

Disturbed epidermal sterol metabolism has been recently found in patients with psoriasis [9], though the nature of the disturbance appears different from that in EFA deficiency. Studies of PG levels in psoriatic skin have revealed conflicting results. Aso et al [10] found decreased PGE_1, $PGF_{2\alpha}$ levels in psoriatic epidermis compared with uninvolved skin whereas Hammerstrom et al [11] found elevated levels of PGE_2 and $PGF_{2\alpha}$ in psoriatic epidermis and an accumulation of arachidonic acid.

In this study care was taken to minimize ingestion of the linoleic acid by restraining the mice after treating and by individual caging. Linoleic acid was applied to the midback and the mice were not observed to be able to groom this area easily. It is however still possible that some of the linoleic acid may have been ingested and we were seeing a partial effect of oral ingestion and a partial effect from percutaneous absorption.

Our results show that:

1. EFA deficiency in the hairless mice was associated with increased DNA synthesis, epidermal hyperproliferation, acanthosis, hyperkeratosis and hypergranulosis.

2. There was a significant lowering of epidermal PGE and PGF in the skin of EFA deficient mice presumably from decreased availability of precursor acids.

3. Linoleic acid reduced the high epidermal DNA synthesis, histologically corrected the acanthosis and reduced the hypergranulosis and hyperkeratosis of EFA deficiency.

4. Linoleic acid partially corrected epidermal PGE and PGF levels in the EFA deficient mice.

REFERENCES

1. Burr GO, Burr MM: A new deficiency disease produced by the rigid exclusion of fat from the diet. J Biol Chem 82:345–367, 1929
2. Lowe NJ, Stoughton RB: Essential fatty acid deficient hairless mouse, a model of chronic epidermal hyperproliferation. Br J Dermatol 96:155–162, 1977
3. Ramalingaswami V, Sinclair HM: Pathological changes in the rat in deficiency of essential fatty acids. Br J Dermatol 65:1–22, 1953
4. Menton DN: The effects of essential fatty acid deficiency on the skin of the mouse. Am J Anat 122:337–355, 1968
5. Kingery FAJ, Kellum RE: Essential fatty acid deficiency: Histochemical changes in the skin of rats. Arch Dermatol 91:272–279, 1965
6. Nasr AN, Shostak S: Mitotic activity in the skin of mice deficient in essential fatty acids. Nature 207:1395, 1965
7. Prottey C, Hartop PJ, Press M: Correction of the cutaneous manifestations of essential fatty acid deficiency in man by application of sunflower seed oil to the skin. J Invest Dermatol 64:228–234, 1975
8. Ziboh VA, Hsia SL: Effects of prostaglandin E_2 on rat skin: Inhibition of sterol ester biosynthesis and clearing of scaly lesions in essential fatty acid deficiency. J Lipid Res 13:458–467, 1972
9. Cooper MF, McGrath H, Shuster S: Epidermal lipid metabolism in psoriasis and lichen simplex. Br J Dermatol 94:369–378, 1976
10. Aso K, Deneau DG, Krulig L, Wilkinson D, Farber EM: Epidermal synthesis of prostaglandins and their effect on levels of cAMP. J Invest Dermatol 64:326–331, 1975
11. Hammerstrom S, Hamburg M, Samuelsson B, Duell EA, Stawitski M, Voorhees JJ: Increased concentrations of non-esterified arachidonic acid, 12 L hydroxy 5, 8, 10, 14-eicosatetraenoic acid, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ in epidermis of psoriasis. Proc Natl Acad Sci USA 72:5130–5134, 1975
12. Lowe NJ: Essential fatty acid deficient hairless mouse: The effects of topical agents on the epidermis. Br J Dermatol 97:39–47, 1977
13. Jaffe BM, Behrman HR: Prostaglandins E, A, and F. Methods of Hormone Radioimmunoassay. New York, Academic, 1974, p 19–34
14. Levine L, Gutierrez-Cernosek RM, Van Vunakas H: Specificities of prostaglandins F, $F_{1\alpha}$ and $F_{2\alpha}$ antigen-antibody reactions. J Biochem 246:6782–6785, 1971
15. Gutierrez-Cernosek RM, Morril LM, Levine L: Prostaglandin $F_{2\alpha}$ levels in peripheral sera of man. Prostaglandins 1:71–80, 1972
16. Caldwell BV, Burnstein S, Brock WA, Speroff L: Radioimmunoassay of the F prostaglandin. J Clin Endocrinol 33:171–177, 1971
17. du Vivier A, Bible R Jr, Mikuriya RK, Stoughton RB: An animal model for screening drugs for antipsoriatic properties using hydroxyapatite to isolate DNA rapidly from the epidermis. Br J Dermatol 94:1–6, 1976
18. Prottey C: Essential fatty acids and the skin. Br J Dermatol 94:579–587, 1976
19. Fulco AJ, Mead JF: Metabolism of essential fatty acids. J Biol Chem 234:1411–1416, 1959
20. Van Dorp DA: Essential fatty acids and prostaglandins, XXIVth International Congress of Pure and Applied Chemistry, Vol 2. Butterworth, London, pp 117–136
21. De Thomas ME, Brenner RR, Peluffo RO: Position of eicosatrienoic acid in phosphatidylcholine and phosphatidyl ethanolamine from rats in essential fatty acid deficiency. Biochem Biophys Acta 70:472–474, 1963
22. Bem JL, Greaves MW: Prostaglandin E_1 effects on epidermal cell growth in vitro. Arch Dermatol Forsch 251:34–41, 1974
23. Eaglestein WH, Weinstein GD: Prostaglandins and DNA synthesis in human skin. Possible relationship to ultraviolet light effects. J Invest Dermatol 64:386–389, 1975
24. Lupulescu AP: Cytologic and metabolic effects of prostaglandins on rat skin. J Invest Dermatol 66:281, 1976
25. Lowe NJ, Stoughton RB: Effects of topical prostaglandin E_2 analogue on normal hairless mouse epidermal DNA synthesis. J Invest Dermatol 68:134–137, 1977
26. Snyder DS: Effect of topical indomethacin on UVR induced redness and prostaglandin E levels in sunburned guinea pig skin. Prostaglandins 11:631–643, 1976